An Ivermectin-Sensitive Glutamate-Gated Chloride Channel from the Parasitic Nematode Haemonchus contortus

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ABSTRACT

Nematode glutamate-gated chloride channels are targets of the macrocyclic lactones, the most important group of anthelmintics available. In Xenopus laevis oocytes, channels formed by the GluClα3B subunit from the parasite Haemonchus contortus were more sensitive to L-glutamate ($EC_{50} = 27.6 \pm 2.7 \ \mu$M) than those formed by the homologous subunit from Caenorhabditis elegans ($EC_{50} = 2.2 \pm 0.12$ mM). Ibotenate was a partial agonist ($EC_{50} = 87.7 \pm 3.5 \ \mu$M). The H. contortus channels responded to low concentrations of ivermectin (estimated $EC_{50} = {\sim}0.1 \pm 1.0$ nM), opening slowly and irreversibly in a highly cooperative manner: the rate of channel opening was concentration-dependent. Responses to glutamate and ivermectin were inhibited by picrotoxinin and fipronil. Mutating an N-terminal domain amino acid, leucine 256, to phenylalanine increased the EC_{50} for L-glutamate to $92.2 \pm 3.5 \ \mu$M, and reduced the Hill number from 1.89 ± 0.35 to 1.09 ± 0.16. It increased the K_d for radiolabeled ivermectin binding from 0.35 ± 0.1 to 2.26 ± 0.78 nM. Two other mutations (E114G and V235A) had no effect on L-glutamate activation or ivermectin binding: one (T300S) produced no detectable channel activity, but ivermectin binding was similar to wild-type. The substitution of any aromatic amino acid for Leu256 had similar effects in the radioligand binding assay. Molecular modeling studies suggested that the GluCl subunits have a fold similar to that of other Cys-loop ligand-gated ion channels and that amino acid 256 was unlikely to play a direct role in ligand binding but may be involved in mediating the allosteric properties of the receptor.

Nematode glutamate-gated chloride channels (GluCl) have attracted considerable attention as the site of action of the avermectin/milbemycin class of anthelmintics (Arena et al., 1992; Wolstenholme and Rogers, 2005). The mode of action of these drugs is very interesting: they act as almost irreversible, long-acting agonists both at recombinant and native GluCl (Cully et al., 1994; Brownlee et al., 1997; Holden-Dye and Walker, 2006). In addition, at concentrations lower than those that cause direct activation of the channels, they potentiate the response to submaximal concentrations of L-glutamate (Cully et al., 1994; Vassilatis et al., 1997; Forrester et al., 2003, 2004). The GluCl are members of the Cys-loop ligand-gated ion channel family, and there is a small family of genes encoding GluCl subunits in both free-living and parasitic nematodes (Yates et al., 2003; Williamson et al., 2007). One member of this gene family that is widely conserved is avr-14, which has been found in Caenorhabditis elegans, Haemonchus contortus, Ascaris suum, Cooperia oncophora, the cyathostomins (parasites of horses), and the filarial parasites infecting humans and companion animals (Laughton et al., 1997; Jagannathan et al., 1999; Dent et al., 2000; Njue et al., 2004; Yates and Wolstenholme, 2004; Tandon et al., 2006). The gene is alternatively spliced in most of these species to yield two subunits, GluClα3A and GluClα3B, that share a common N-terminal ligand-binding domain but differ in the C-terminal channel-forming domains. Previous studies on these subunits have indicated that the GluClα3B subunit can form channels that bind radiolabeled ivermectin (22,23-dihydroavermectin B1a) (Campbell et al., 1983) with very high affinity (Cheeseman et al., 2001) and are activated by L-glutamate- and ivermectin when expressed alone, but that the GluClα3A subunit cannot (Dent et al., 2000; Njue et al., 2004; Yates and Wolstenholme, 2004; Tandon et al., 2006).

The expression pattern of this gene has been studied using reporter gene constructs, which would not distinguish between the two splice variants in C. elegans (Dent et al., 2000),

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ABBREVIATIONS: GluCl, glutamate-gated chloride channel; OR2, oocyte Ringers 2 solution; Ptx, picrotoxinin; Fip, fipronil; Ptx, picrotoxinin.

and specific antibodies against the conserved N-terminal and variable C-terminal regions in *H. contortus* (Jagannathan et al., 1999; Portillo et al., 2003). In both species, gene expression was detected in extrapharyngeal neurons in the head, sensory neurons, and ventral cord motor neurons and in *H. contortus* expression of the GluClα3A subunit was also detected in amphid neurons with the GluClα3B subunit found in three putative pharyngeal neurons. Mutations in *avr-14* have been associated with avermectin resistance in *C. elegans* and *C. oncophora* (Dent et al., 2000; Njue et al., 2004). In *C. oncophora*, three amino acid differences, E114G, V235A, and L256F, were found between the GluClα3 subunits from avermectin-susceptible and -resistant worms. The resultant recombinant channels from the resistant isolate were 3.2-fold less sensitive to L-glutamate and 2.6-fold less sensitive to ivermectin than those from the susceptible isolate, and this difference was wholly due to the L256F polymorphism alone (Njue et al., 2004). Avermectin resistance has become a major problem in agricultural parasites (Wolstenholme et al., 2004), notably in *H. contortus*, and may be emerging in human parasites (Osei-Atweneboana et al., 2007). Because changes in GluClα3B have been implicated in resistance, we studied the ability of glutamate and ivermectin to activate the channels it forms, as well as the effect of candidate resistance mutations, identified both in GluCl and in other ligand-gated chloride channels, on its interactions with the drug.

Materials and Methods

Materials. All common chemicals and biochemicals were from Sigma (Poole, Dorset, UK). The mMessage mMachine T7 RNA transcription kit was from Ambion Inc. (Huntingdon, UK). Mature female *Xenopus laevis* frogs were supplied by Blades Biologicals (Kent, UK). Restriction enzymes were from Promega (Southampton, UK). Fipronil was a gift from Aventis CropScience (Frankfurt, Germany).

Site-Directed Mutagenesis and cRNA Synthesis. Site-directed mutagenesis was carried out using Stratagene QuikChange mutagenesis kit (Stratagene, La Jolla, USA). Plasmid DNA (10 μg) was linearized using the restriction enzyme XbaI, extracted with phenol/chloroform, and precipitated with ethanol at −20°C overnight. After centrifugation, the ethanol was completely removed, the pellet resuspended in nuclease-free water (10 μl), and stored frozen at −20°C. The linear template DNA (1 μg) was used with the mMessage mMachine kit to generate RNA by the method of in vitro transcription. The RNA was diluted to 1 μg/μl and stored in working aliquots at −80°C.

Expression in *X. laevis* Oocytes and Two-Electrode Voltage Clamp Recordings. Mature female *X. laevis* were killed by terminal anesthesia in 0.2% (w/v) benzocaine solution [10% (w/v) in ethanol, diluted 1:50 with water]. Ovarian lobes were dissected out under sterile conditions and placed in OR2 calcium-free buffer (82 mM NaCl, 1 mM MgCl₂, 2 mM KCl, and 5 mM HEPES, pH 7.5). The lobes were then teased into small clumps of oocytes and washed several times with OR2 buffer. Oocyte clumps (5 ml) were gently agitated with 10 ml of type 1A collagenase solution (1.5 mg/ml in OR2) for 75 min at 18°C, before washing with OR2 buffer and transferral to ND96 buffer [96 mM NaCl, 1 mM MgCl₂, 2 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES, and 1% (v/v) fetal bovine serum, pH 7.5]. Remaining clumps of oocytes were removed and large, disassociated oocytes were selected for injection. Selected oocytes were allowed to recover overnight with gentle agitation at 18°C, and then microscopically examined for injection suitability. Fifty nanograms of *Hc*GluClα3B RNA (in 50 nl of nuclease-free water) was injected into each oocyte (approximately 30 in total) under sterile conditions.

The injected oocytes were then incubated in sterile ND96 at 18°C, with gentle agitation for 3 to 6 days and medium changes at 24-h intervals.

Oocytes were placed in a poly(methyl methacrylate) chamber and perfused with Ringer solution (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5). Two glass microelectrodes (0.5–5-mΩ resistance) were attached to HS-2A preamplifiers (Molecular Devices, Sunnyvale, CA) and inserted through the oocyte membrane. The resting membrane potential was displayed on a GeneClamp 500B voltage clamp amplifier (Molecular Devices). Typically, an oocyte resting membrane potential difference was seen to be −30 to −50 mV. Current was injected through one microelectrode to increase this value to −80 mV, where it was held (clamped) throughout the perfusion experiments.

L-Glutamic acid, ibotenic acid, aspartic acid, glycine, GABA, histamine, and 5-hydroxytryptamine were all dissolved in Ringer solution at high concentration (100 mM–1 M), adjusted to pH 7.5, and diluted appropriately before perfusion through the oocyte chamber for 5 to 10 s. A washing period of 5 min was allowed between each perfusion event. Ivermectin, picrotoxinin (Ptx), and fipronil (Fip) were dissolved at high concentration (100 mM–1 M) in dimethyl sulfoxide and diluted appropriately in Ringer solution to a final concentration of 0.1% dimethyl sulfoxide. A different chamber (that was soaked in ethanol overnight after each use) was used for perfusion experiments with these compounds. In blocking experiments, Ptx and fipronil were perfused for 2 min before L-glutamate administration.

Expression in COS-7 Cells and Radioligand Binding Assays. A full-length *H. contortus* cDNA encoding GluClα3B was subcloned into pcDNA3.1. One day before transfection, tissue-culture dishes were seeded with COS-7 cells at a density so that they were 90 to 95% confluent at the time of transfection. Transfection of the cDNA was accomplished using Lipofectamine 2000, and cells incubated at 37°C in an incubator with 5% CO₂ for 18 to 48 h before harvesting membrane protein. The culture medium was removed by aspiration from the plate and the cells washed three times with room temperature 50 mM HEPES with 0.01% (w/v) sodium azide, pH 7.4. Cells were harvested with a cell scraper in 5 ml of the 50 mM HEPES with 0.01% (w/v) sodium azide and placed in a 15-ml universal centrifuge tube. The cells were further disrupted by sonication in three 10-s bursts on medium power.

The medium was made up to 10 ml with more 50 mM HEPES with 0.01% sodium azide and added to a 10-ml ultracentrifuge tube. The cells were centrifuged at 75,000*g* for 30 min at 4°C. The supernatant was discarded and the cells resuspended in 1 ml of 50 mM HEPES with 0.01% (w/v) sodium azide and 0.2 mM phenylmethylsulfonyl fluoride. [³H]Ivermectin binding studies were carried out in 5 ml of polypropylene tubes. Membrane preparations were incubated with [³H]ivermectin [specific activity, 1.44 TBq/mmol; a custom synthesis by Amersham International (now GE Healthcare), Chalfont St. Giles, Buckinghamshire, UK] in 50 mM HEPES, pH 7.4, with 10 μg of protein. Nonspecific binding was determined by binding in the presence of 10 μM unlabeled ivermectin, and the final assay volume was made up to 1 ml with 50 mM HEPES. The reactions were incubated at room temperature for 2 h. The binding reaction was quashed by dilution with 3 ml of unlabeled HEPES wash buffer with 0.01% (w/v) sodium azide and 0.25% (v/v) Triton X-100. The halted reactions were filtered through type A/B glass fiber filters (Whatman, Kent, UK) that had been presoaked in 3% (v/v) polyethylenimine in wash buffer. The filters were rinsed three times in wash buffer and placed in individual minivials to which 5 ml of OptiPhase Safe was added (PerkinElmer Life and Analytical Sciences, Bucks, UK). Bound radioactivity was determined by liquid scintillation spectrometry.

Molecular Modeling. A BLAST search (Altschul et al., 1997) through NCBI was used to identify structures homologous to the GluCl by searching the structural database of protein sequences in the Protein Data Bank (Berman et al., 2000). A parallel comparison

was carried out using the MODELLER program. The crystal structure of the acetylcholine binding protein from *Lymnea stagnalis* (Protein Data Bank code 1I9BA) was selected as a template for the homology modeling for the GluClα3B subunit. This protein was the top alignment from the MODELLER search and the best match over 50 amino acids from the BLAST search. The amino acid sequences of GluClα3B and 1I9B were aligned using the align2d command in MODELLER, which aligns multiple sequences using a pairwise progressive alignment algorithm: the two polypeptides were 21% identical, with a similarity of 53%. The alignments were checked for any deletions or insertions in the structurally conserved and structurally variable regions between the template and target sequences. Homology models of wild-type and mutant Gluclα3B were constructed using Modeler 9v2 (http://salilab.org/modeller/modeller.html), based upon the individual sequence/template alignments. Modeler was used to model 3D structure of protein by satisfaction of spatial restraints on the structure of the amino acid sequence. All structures and models were visualized using the protein explorer 2.80 (http://www.umass.edu/microbio/chime/pe_beta/pe/protexpl/frntdoo2.htm). The stereochemical, volume, and surface properties of the models were evaluated using modeler and procheck (Laskowski et al., 1993; http://www.biochem.ucl.ac.uk/~roman/procheck/procheck.html).

Results

The cDNAs encoding the *H. contortus* GluClα3A, α3B (Jagannathan et al., 1999), and β (Delany et al., 1998) subunits and the *C. elegans* GluClα3B (Laughton et al., 1997) subunit were transcribed into cRNA in vitro and microinjected into *X. laevis* oocytes. After 2 to 6 days of incubation, oocytes were
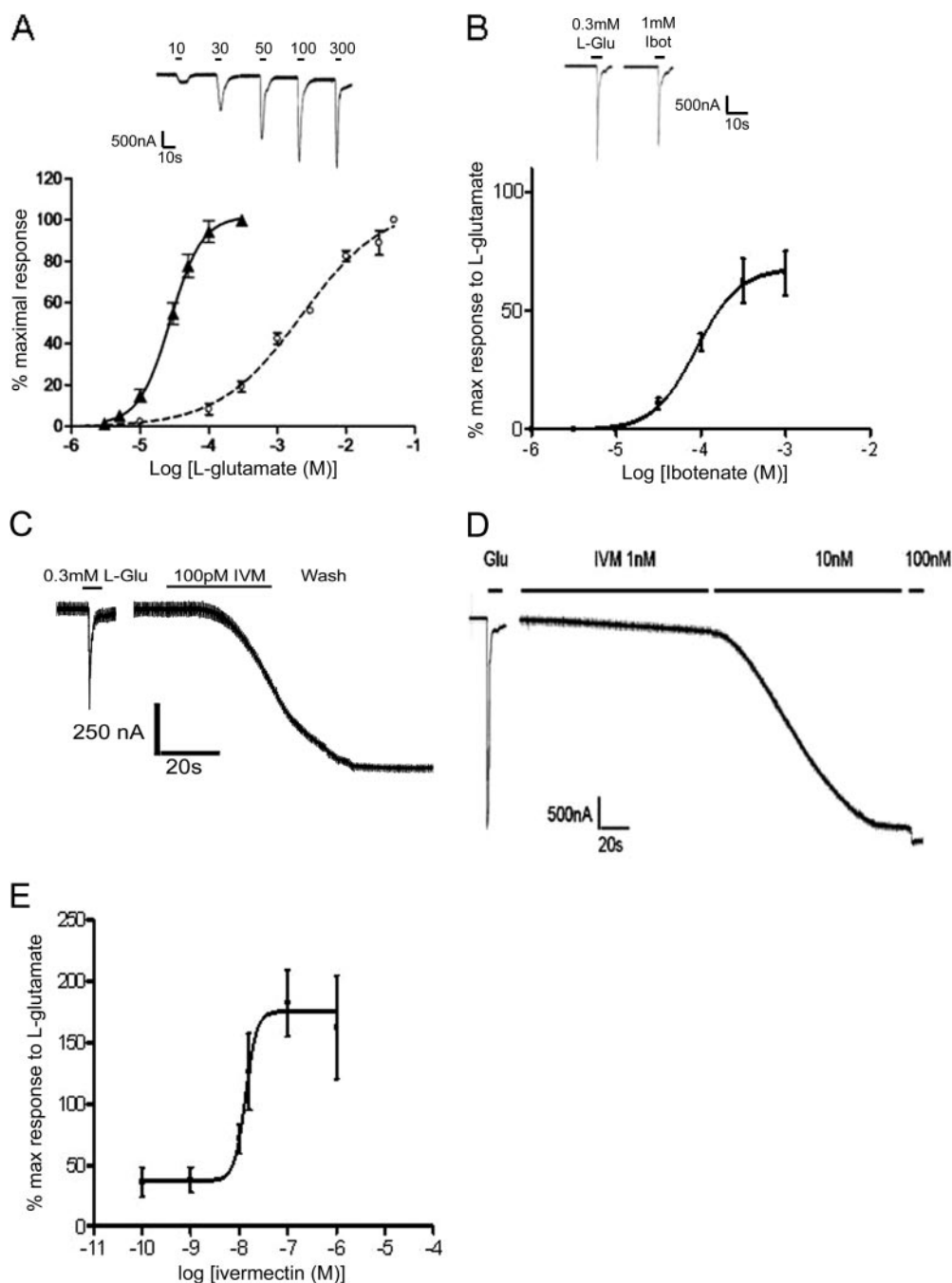

Fig. 1. Action of agonists on the GluClα3B channels. A, dose-response curve of L-glutamate applied to the *H. contortus* and *C. elegans* GluClα3B channels expressed in *Xenopus* oocytes, measured using two-electrode voltage clamp. ▲, *H. contortus* channel; ○, *C. elegans* channel. An example of a dose-response curve from the *H. contortus* channel is shown above the graph: the numbers represent the concentration of L-glutamate applied (μM). B, action of ibotenate on the *H. contortus* channel. Typical responses to the maximal doses of L-glutamate (300 μM) and ibotenate (1 mM) are shown above a dose-response curve for ibotenate, normalized to the maximal response to L-glutamate. C, action of ivermectin on the *H. contortus* channel. The slow and irreversible opening of the channel is illustrated, compared with the response to 300 μM L-glutamate. D, concentration-dependent responses to three concentrations of ivermectin are shown, illustrating the decrease in the rate of channel opening with increasing concentration, along with the response to 300 μM L-glutamate. E, dose-response curve for the action of ivermectin on the *H. contortus* GluClα3B channel.

voltage-clamped at -80 mV and tested for responses to 1 mM L-glutamate. Responses were detected only from oocytes injected with GluClα3B cRNA, either alone or in combination with the other cRNAs (Fig. 1). No responses were detected from either uninjected oocytes or control oocytes into which

water was injected: both controls were carried out on three to four oocytes from each experimental batch (a total of >40 oocytes from >10 frogs). The shape and amplitude of the responses obtained in response to a range of concentrations of L-glutamate in oocytes injected with α3B with either α3A
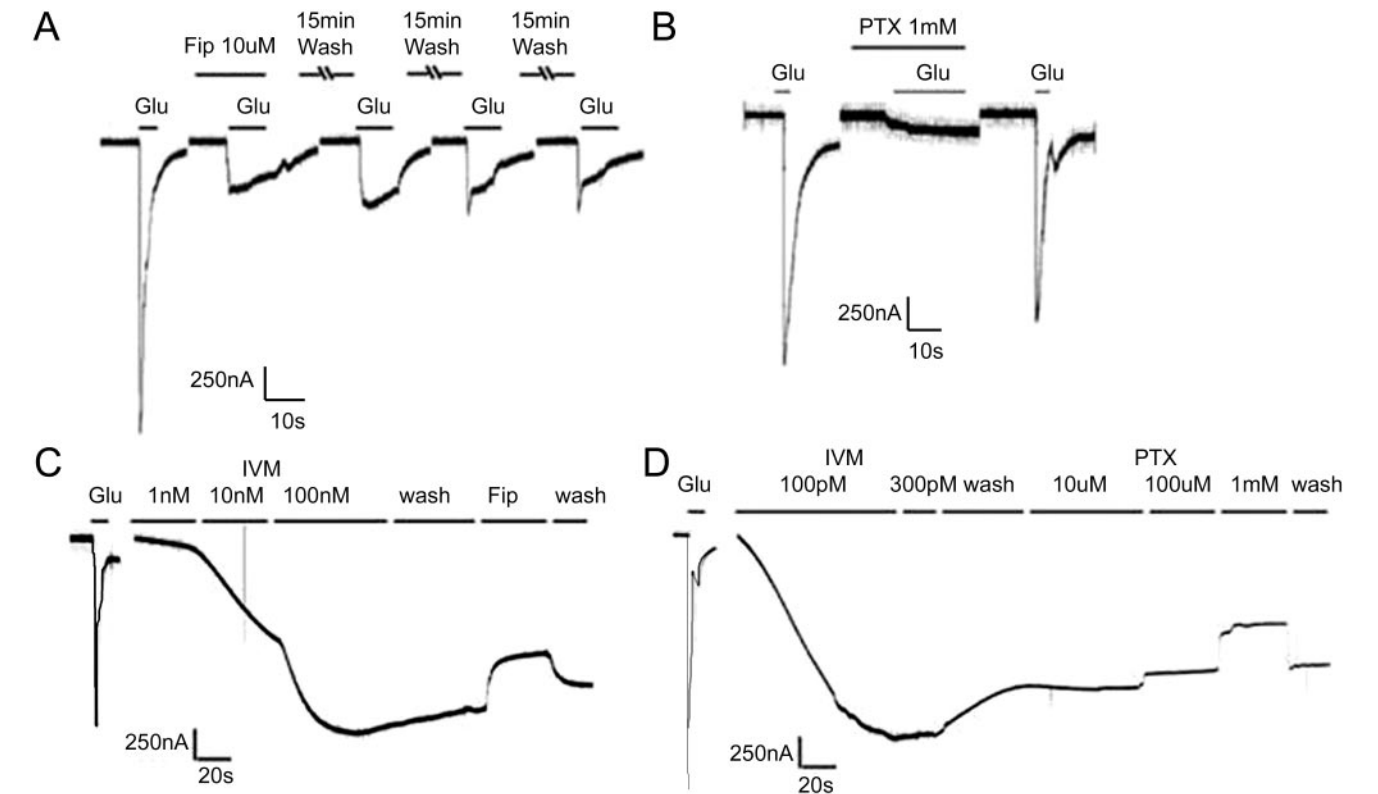

Fig. 2. Effect of noncompetitive antagonists on the *H. contortus* GluCl α3B channels. A, fipronil (Fip) at 10 μM partially blocks the response to 100 μM L-glutamate and does not wash out. B, picrotoxinin (Ptx) at 1 mM almost completely blocks the L-glutamate response (100 μM) in a reversible manner. C, Fip (10 μM) partially and reversibly blocks the ivermectin-induced channels. D, Ptx blocks the ivermectin-induced channels in a concentration-dependent and partially reversible manner.

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1         11        21        31        41        51
MRNSVPLATRIGPMLALICTVSTIMSAVEAKRKLKEQEIIQRILNNYDWRVRPRGLNASW

61        71        81        91        101       111■
PDTGGPVLVTVNIYLRSISKIDDVNMEYSAHFTFREEWVDARLAYGRFEDESTEVPPFVV
                                                          G

121       131       141       151       161       171      ▲
LATSENADQSQQIWMPDTFFQNEKEARRHLIDKPNVLIRIHKDGSILYSVRLSLVLSCPM

181       ▲         201       211       221       231 ■
SLEFYPLDRQNCLIDLASYAYTTQDIKYEWKEQNPVQQKDGLRQSLPSFELQDVVTKYCT
                                                          A

241       251  ■    261       271       281       291         ■
SKTNTGEYSCARVKLLLRREYSYYLIQLYIPCIMLLVVSWVSFWLDKDAVPARVSLGVTT
          F                                                  S

301       311       321       331       341       351
LLTMTTQASGINSKLPPVSYIKAVDVWIGVCLAFIFGALLEYAVVNYYGRKEFLRKEKKK

361       371       381       391       401       411
KTRLDDCVCPSERPALRLDLSNYRRRGWTPLNRLLDMLGRNADLSRRVDLMSRITFPSLF

421       431
TAFLVFYYSVYVKQSNLD
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Fig. 3. Primary amino acid sequence of the *H. contortus* GluClα3B subunit. The positions at which the mutations were introduced are indicated by ■, and the changes are shown underneath the sequence. The signal peptide is in italics, the membrane-spanning regions are shaded in gray, and the position of the cysteine residues that form the "Cys-loop" are shown by ▲.

or the β subunits were indistinguishable from those obtained from cells expressing only the α3B subunit (data not shown), so the properties of the homomeric α3B channels were examined in more detail. L-Glutamate caused a rapidly desensitizing response, with an EC$_{50}$ of 27.6 $\pm$ 2.7 μM and a Hill number of 1.89 $\pm$ 0.35, suggesting that more than one molecule of glutamate activated the channel with positive cooperativity. The *H. contortus* GluClα3B channels were much more sensitive to L-glutamate than those formed by the orthologous subunit from *C. elegans* (Fig. 1A), which had an EC$_{50}$ of 2.2 $\pm$ 0.12 mM and a Hill number of 0.72 $\pm$ 0.08, with no evidence of positive cooperativity.

Nematodes possess a large family of ligand-gated anion channels, including those gated by GABA and 5-hydroxytryptamine. In addition, histamine-gated chloride channels have been identified in insects, and vertebrates contain glycine-gated chloride channels. We therefore treated the *H. contortus* GluClα3B channels with 1 and 10 mM GABA, glycine, 5-hydroxytryptamine, histamine, and L-aspartate. None of these compounds induced any measurable channel activity, except that occasionally very small responses were seen upon application of 10 mM L-aspartate (data not shown). By contrast, the conformationally constrained glutamate analog, ibotenic acid, caused a robust, dose-dependent, and fully reversible activation of the channel (Fig. 1B), with an EC$_{50}$ of 87.7 $\pm$ 3.5 μM and a Hill number of 1.70 $\pm$ 0.36. The maximal responses observed on ibotenate application were 69 $\pm$ 5.2% of those seen with L-glutamate, indicating that it is a partial agonist at this receptor.

Ivermectin was also able to activate the HcGluClα3B channels, although, as has been previously shown for other nematode GluCl (Cully et al., 1994; Dent et al., 1997, 2000; Vassilatis et al., 1997; Horoszok et al., 2001; Pemberton et al., 2001; Forrester et al., 2004; Njue et al., 2004), the activation was slow and irreversible (Fig. 1C). These properties made it difficult to determine an accurate EC$_{50}$ for this activation, and this problem was compounded by our observation that it was not the size of the response, in terms of the peak current, that was dose-dependent, but the rate at which the channels opened and that current was obtained (Fig. 1D). The lowest concentration of ivermectin that produced robust responses was 0.1 nM, and application of concentrations $\geq$1

nM reliably induced channel opening. With the limitations described, we estimated an EC$_{50}$ for ivermectin of 0.1 $\pm$ 1.0 nM at this receptor, with a very high apparent Hill number (>6) (Fig. 1E).

Picrotoxinin and fipronil have previously been reported to antagonize the glutamate responses at some, but not all, nematode GluCl. We found that picrotoxinin would reversibly block channels opened in response to a submaximal concentration of L-glutamate in a dose-dependent manner (Fig. 2A). Fipronil (10 μM) blocked the glutamate-induced channels by 74 $\pm$ 7%, although in this case, the block was not reversed, even by extensive (30 min) washing (Fig. 2B). Picrotoxinin (1 mM) also reversibly reduced a maximal ivermectin response by up to 56 $\pm$ 7% (Fig. 2C), and fipronil (100 μM) also partially reduced a similar response by 35% (Fig. 2D). In this case, the fipronil block was reversible and terminated by washing.

To test whether changes in the GluClα3B subunit could contribute to ivermectin resistance, we created mutant forms of the GluClα3B cDNA that contained the three potential ivermectin resistance-associated mutations identified by Njue et al. (2004), plus an additional mutation, T300S, previously observed in ivermectin-resistant *H. contortus* in our laboratory (Jagannathan, 1998) (Fig. 3). These were then expressed in the *X. laevis* oocyte system, and the responses to glutamate were measured. Two of the mutants, E114G and V235A, produced channels practically identical to wild-type (Fig. 4 and Table 1), and T300S failed to produce any channels at all. When T300S was coexpressed with wild-type channels at a ratio of 1:1 (based on the amount of injected cRNA), the channels produced were identical to wild-type (data not shown). Attempts to determine whether this was due to a failure of this subunit to express correctly were unsuccessful. The final mutation, L256F, caused a shift in the dose-response curve to L-glutamate to the right, increasing the EC$_{50}$ to 92.2 $\pm$ 3.5 μM, and also resulted in a significant loss of cooperativity in the receptor, with the Hill number decreasing to 1.09 $\pm$ 0.16.

Because obtaining good quantitative data using ivermectin in the oocyte system had proved problematic, we turned to radioligand binding assays to study the effects of the mutations on the interaction between ivermectin and the GluClα3B receptor (Cheeseman et al., 2001). The mutant subunits were expressed in COS-7 cells, and their ability to bind [^{3}H]ivermectin was measured (Fig. 5). In these experiments, the wild-type subunit bound the drug with a K_d of 0.35 $\pm$ 0.1 nM. In this system, the T300S mutant was expressed successfully and was able to bind the drug, with a K_d
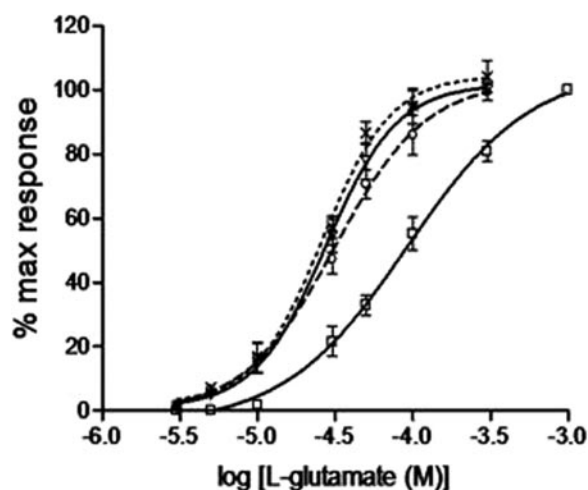

Fig. 4. Dose-response curves for L-glutamate at mutant *H. contortus* GluClα3B channels. $\triangle$, wild-type; $\times$, V235A; $\bigcirc$, E114G; $\square$, L256F.

TABLE 1
The effects of ivermectin resistance-associated mutations on the ability of glutamate to activate *H. contortus* GluClα3B channels
All values are given as the mean $\pm$ S.E.M.

Mutation	EC$_{50}$ for L-Glutamate	Hill Number	Number of Measurements
	μM		
Wild type	27.6 $\pm$ 2.7	1.89 $\pm$ 0.35	7
E114G	31.5 $\pm$ 3.2	1.48 $\pm$ 0.31	6
V235A	26.2 $\pm$ 2.5	1.97 $\pm$ 0.35	6
L256F	92.2 $\pm$ 3.5***	1.09 $\pm$ 0.16**	8
T300S	No channels		

** $p < 0.01$.
*** $p < 0.001$.

of 0.76 ± 0.25 nM. The E114G and V235A mutants again gave results very similar to those of wild-type, whereas the L256F mutant showed a significantly increased K_d of 2.26 ± 0.78 nM. We therefore introduced some additional mutations at this position, finding that the substitution of any aromatic amino acid for Leu256 caused a significant increase in the K_d for ivermectin, none of which was significantly different from L256F (Table 2), but that the more conservative substitution
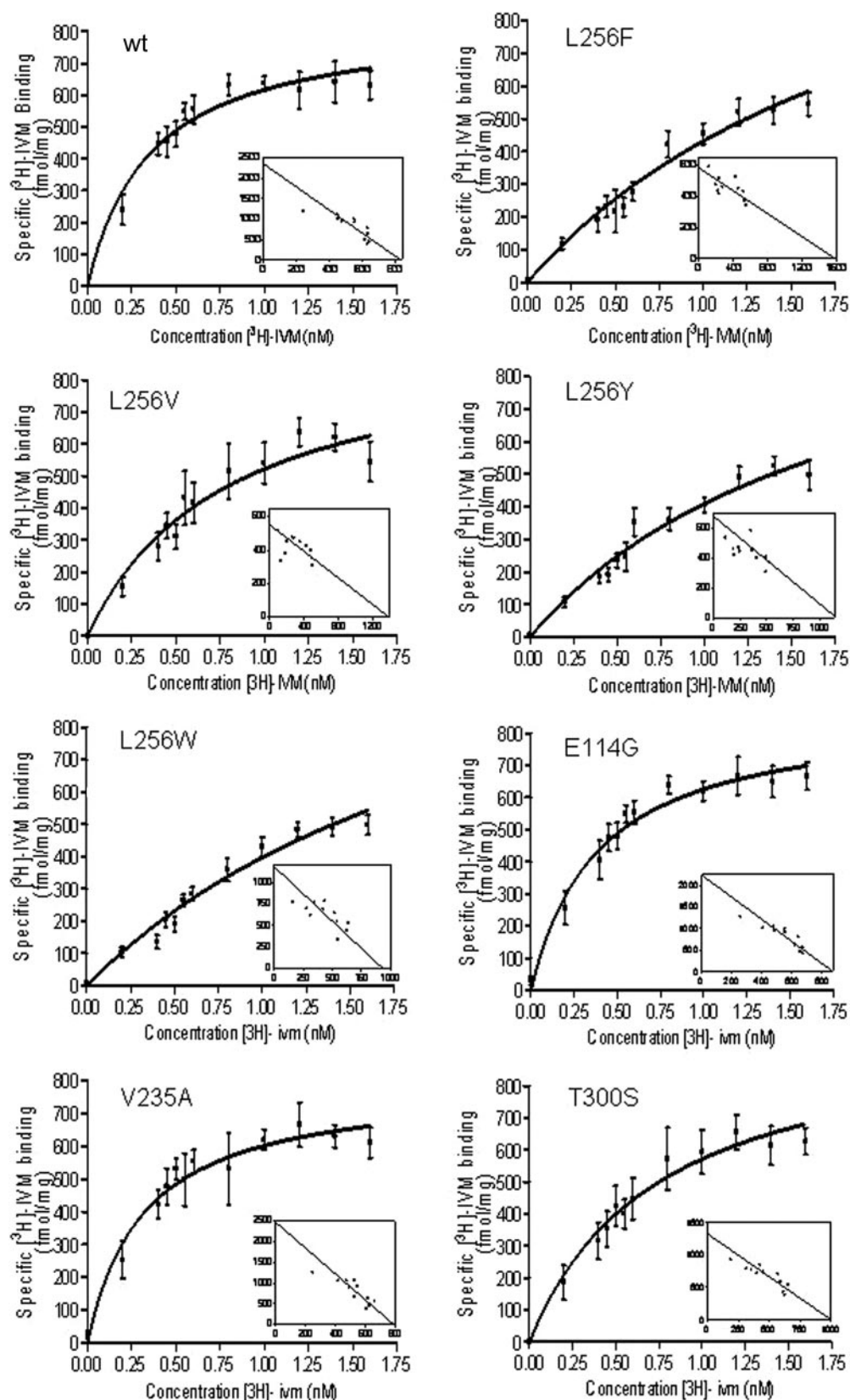

Fig. 5. Radioligand binding assays using [³H]ivermectin and membrane preparations from COS-7 cells transfected with wild-type and mutant *H. contortus* GluClα3B. The insets show the Scatchard plots for each mutant.

of a valine had a reduced effect, producing a receptor with a K_d of 0.74 ± 0.24 nM.

In an attempt to gain some insight into the possible mechanisms via which mutations at amino acid residue 256 might cause the observed changes in receptor properties, we attempted to model the three-dimensional structure of the extracellular domain of the GluClα3B subunit, using the known structure of the soluble acetylcholine-binding protein from *Lymnaea stagnalis* (Brejc et al., 2001) as a template. This protein has been widely used as a template for modeling the structures of members of the cys-loop ligand-gated ion channel superfamily, although its sequence similarity to the GluCl (53%) is rather lower than to the nicotinic receptors. The model of the wild-type extracellular domain is shown in Fig. 6. We looked at the effects of the mutations at position 256 on this model. The wild-type amino acid, leucine, is adjacent to a relatively large number of other amino acid residues and in the pentameric structure is found in the center of the outside face of the extracellular channel (Fig. 6). The two residues that are most closely associated with the Leu256 are Val235, which is interesting because the V235A mutation was found in association with L256F, and Leu193. In the wild-type channel, the leucine seems to be neither attracted nor repulsed by any of its neighbors, in that they all possess relatively unreactive side chains. The modeling of the L256F substitution results in an obvious interaction with the neighboring polar amino acid Asn191, in which an attraction seems to be occurring. This phenomenon is also seen with the L256V and L245Y mutations, although the L256W has no apparent effect on the Asn191 residue. All of the mutations at position 256 were predicted to cause a change in the orientation of amino acid Lys254. Position 235 is found at the bottom of the β-sandwich in a highly conserved area of β10, the ninth β-strand of the sandwich. On the pentameric structure, it is found on the outside facing edge and interacts primarily with residues on the final β11 strand. The most important of these residues is Leu256. However, the V235A change is not predicted to have any major effect on the surrounding amino acids.

Discussion

Ivermectin and its related compounds are the most important anthelmintics available today; as such, we need to better understand their mode of action and how resistance, already an established problem in veterinary medicine,

might develop. *H. contortus* is an important parasite in which ivermectin resistance has already become widespread; given the possibility that this resistance might be due to changes in the GluCl that are the drug target (Njue et al., 2004; McCavera et al., 2007), we have examined the effects of candidate resistance-associated mutations in this receptor. We have confirmed that the products of the nematode *avr-14* gene can be activated by low concentrations of ivermectin; indeed, the *H. contortus* GluClα3B subunit forms channels activated by much lower concentrations of both the natural ligand, glutamate, and the anthelmintic than does the corresponding subunit from either *C. elegans* or another parasite, *Dirofilaria immitis* (Yates and Wolstenholme, 2004). The reasons for this increased sensitivity, also seen with the GluClα3B subunit of *Cooperia oncophora* (Njue et al., 2004), are not known, and its biological significance is not understood. Unlike in *C. elegans* or *C. oncophora* (Cully et al., 1994; Njue et al., 2004), we could find no evidence of channels formed by, or incorporating, the GluClβ subunit of *H. contortus*. This suggests that the GluCl of different nematode species are rather different, supporting observations that ivermectin has varying effects on nematodes (Behnke et al., 1993; Sheriff et al., 2002; Holden-Dye and Walker, 2006). One factor contributing to the difference between the *C. elegans* and *H. contortus* GluClα3B channels might be that the amino acid residue at position 256 in *C. elegans* is phenylalanine rather than leucine, and the L256F change has been associated with resistance in *C. oncophora*. Although we have confirmed that introducing an aromatic amino acid at this position does alter the response of the channel to both glutamate and ivermectin, the effect is not large enough to explain the difference in EC_{50} for glutamate between the homologous receptors from *H. contortus* and *C. elegans*, which is 2 orders of magnitude. Otherwise, the pharmacology of the *H. contortus* GluClα3B channel is similar to that reported for other GluCl, with ibotenate acting as a partial agonist and picrotoxin acting as a channel blocker. The antagonist affects of fipronil confirm that the fiproles do have activity against nematode ligand-gated chloride channels (Horoszok et al., 2001).

The effects of introducing an aromatic amino acid at position 256 were interesting. Although the apparent efficacy of glutamate and the affinity of ivermectin were both reduced, it was noteworthy that this was accompanied by a change in the shape of the dose-response curve and a reduction in the Hill number, suggestive of a loss of cooperativity rather than a change in the actual binding site of either drug or agonist. Because the expressed channels are homomers and hence possess five possible binding sites, it may be that the region of the subunit around position 256, which is at the C-terminal end of the N-terminal extracellular domain (Fig. 6), is involved in communication between subunits, rather than directly in ligand binding. Whatever the mechanism, our data confirm those of Njue et al. (2004) and that this is a candidate polymorphism associated with ivermectin resistance. We are currently assessing whether or not it is present in resistant isolates of *H. contortus*. So far, we have had no success. The T300S polymorphism, originally found in an ivermectin-resistant isolate of *H. contortus* (Jagannathan, 1998), had little effect on ivermectin binding but seemed to prevent the formation of functional channels. This may be due to a defect in expression, assembly, or trafficking

TABLE 2

The effects of mutations in *H. contortus* GluClα3B on binding of [³H]ivermectin to membrane preparations from transfected COS-7 cells

All the transfections were repeated at least three times. Values shown are the mean ± S.E.M.

Mutation	K_d [³H]Ivermectin Binding	Number of Measurements
	nM	
Wild type	0.35 ± 0.1	8
E114G	0.39 ± 0.07	6
V235A	0.32 ± 0.09	6
L256F	2.26 ± 0.78***	8
L256W	2.51 ± 0.7***	6
L256Y	1.84 ± 0.49***	6
L256V	0.79 ± 0.24*	6
T300S	0.76 ± 0.25	8

* $p < 0.05$.
*** $p < 0.001$.

of the channel, or because the protein turns over much more rapidly than wild-type in the oocyte. Because this amino acid is in the channel-lining M2 domain (Jagannathan et al., 1999), it is possible that the substitution results in a blocked channel that does not allow chloride ions to pass through. However, confirmation that this mutant expresses in the oocyte plasma membrane is necessary to confirm this possibility: it was expressed well in the COS-7 cells because the radiolabeled ivermectin binding was similar (in terms of $B_{\max}$ as well as K_d) to that of wild-type. However, ligand binding does not require the channel to be expressed in the plasma membrane.

The molecular modeling results suggest that the GluCl have the same basic fold as other members of the Cys-loop ligand-gated ion channels, notably the nicotinic acetylcholine receptors. Although the similarity between the GluCl subunit we modeled and the acetylcholine binding protein (53%) was not great enough to permit detailed predictions of the structure of the agonist binding site, it seems likely that this site, as in the nicotinic receptors, is located at the interface between adjacent subunits. The changes in cooperativity that we saw when this residue was mutated may indicate that this region of the protein is involved in mediating allosteric
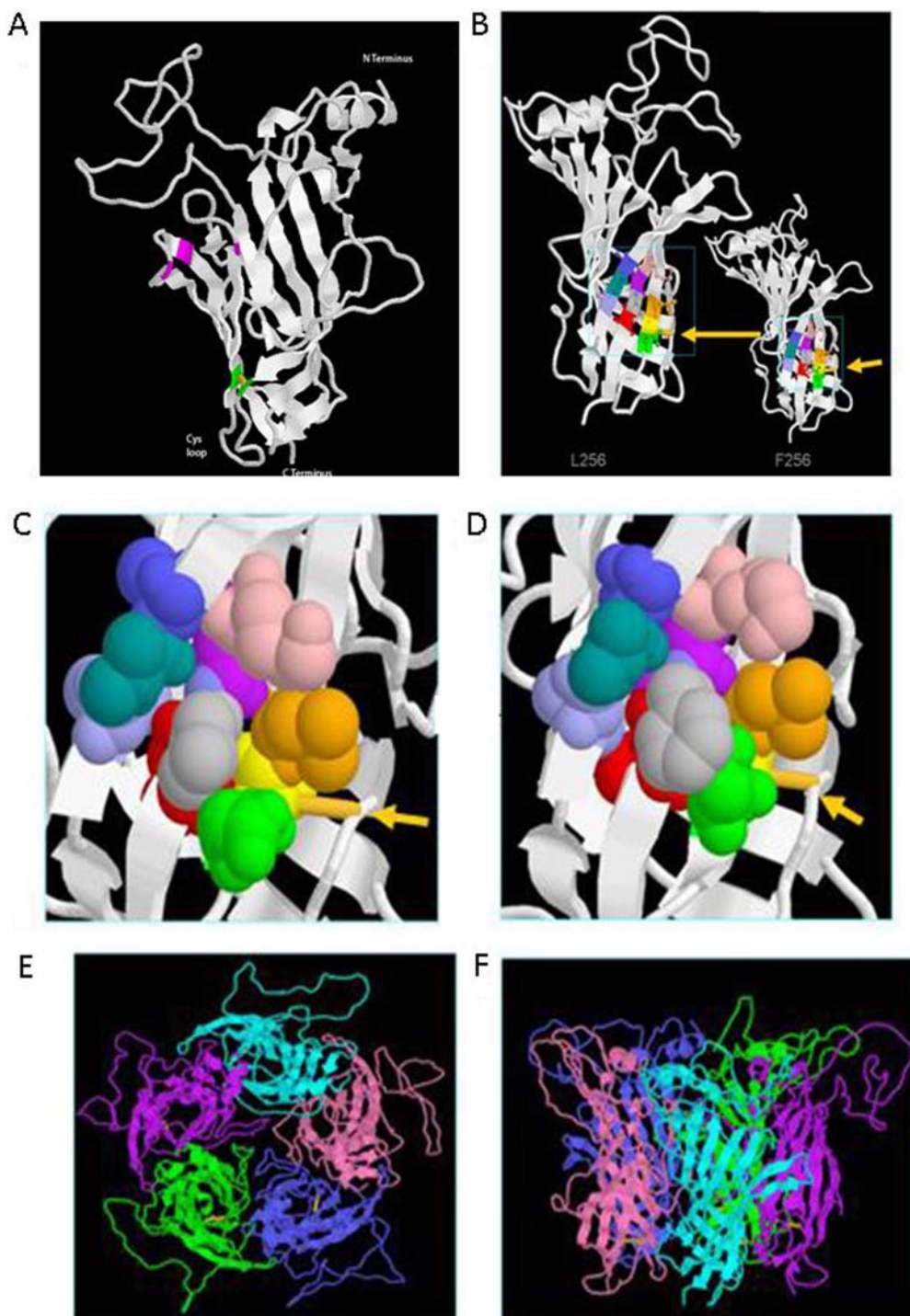

Fig. 6. Molecular modeling of the extracellular domain of a single subunit of *H. contortus* GluClα3B. A, side view of a model of a single GluClα3B subunit, the relative position of the nicotinic ligand binding residues on the primary face is labeled in magenta, the cysteine residues forming the disulfide bridge are shown in green. B, model of the side view of a single GluClα3B subunit. Inset is a model carrying the L256F mutation. Yellow arrows point to the position of the disulfide bridge, and the blue box represents the areas enlarged in C and D. C and D, models of the pentameric structure of the wild-type channel with space filling illustrations of the residues including and contacting position 256. C, leucine at residue 256. D, phenylalanine at position 256. Top (E) and side (F) views of model of the pentameric structure of the wild-type channel. Each subunit is illustrated with a different color.

effects between adjacent binding sites rather than directly in binding ligands.

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